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18.12.89-JP-327725 (15.08.91) C12n-05/20 C12n-15/06 C12p-21/08 C12r-01/91 G01n-33/57

Monoclonal antibody to human interleukin-4 - determined by adding human il-4 to solidified monoclonal antibody, binding second antibody and labelled antibody and determining activity

C91-123114

Monoclonal antibody, 380-1 or 144-6, recognises specifically human interleukin-4(IL-4) and has high affinity to human IL-4. Determining human IL-4 in sample comprises (1) adding a sample contg. human IL-4 to a solidified monoclonal antibody to human IL-4, 380-1 or 144-6 (1st antibody), to bind the monoclonal antibody with human IL-4, (2) binding polyclonal antibody (2nd antibody) to human IL-4 with the bound matter, (3) binding a labelled antibody (3rd antibody) recognising the 2nd antibody with the bound matter obtained in (2), and determining the activity of the labelling substance.

USE/ADVANTAGE - Monoclonal antibody recognises specifically human IL-4 and having high affinity to human IL-4 and utilises the monoclonal antibody, esp. a method for immunoassay using the antibody. By using monoclonal antibody to human IL-4, 380-1 or 144-6, the immuno-assay of human IL-4 can be carried out with very high sensitivity (determination limit is 3.9 pg./ml.), high specificity and high reproducibility. (15pp Dwg.No.0/0)

B(4-B4A3, 4-B4C5, 4-C1G, 5-A4, 11-C7A6, 12-K4A) D(5-H9, 5-H11) B0134

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25.09.89-JP-248867 (+ JP-185813) (15.08.91) A61k-37/54 A61k-39/39 C12n-05/20 C12n-15/06 C12p-21/08 C12r-01/91 G01n-33/57

Antibody producing hybridoma used for immunoassay for TPA detection - obtd. by cell fusion of animal spleen cell or lymphocyte immunised by TPA to myeloma cell

C91-123115

29.06.90-JP-172935

Anti TPA monoclonal antibody which has properties of (a) it binds to TPA competitively to plasminogen activator inhibitor, and (b) it does not neutralize enzyme activity of TPA. The hybridoma which is obtd. by cell fusion of animal spleen cell or lymphocyte immunised by TPA to myeloma cell, and can produce antibody of (1). Thrombolytic drug which is composed by immuno-binding TPA to antibody of (1).

Immunochemical assay for TPA in test sample comprises test sample soln. is reacted to anti-TPA antibody retained on carrier, next, reacted to anti-TPA antibody labelled with labelled cpd., then, activity of labelled cpd. retained on the carrier, or activity of labelled cpd. non-retained on the carrier is assayed, where antibody retained on the carrier and antibody labelled by labelled cpd. are 2 kinds of antibodies that have no-duplicative antigen determining sites each other, and one is antibody of (1).

USE/ADVANTAGE - Anti-TPA MoAb can binds to TPA without injuring fibrin solubilising effect of TPA, also can block inhibiting effect to TPA activity by PAI-I. By combination use with TPA, more effective and low side effective solubilising and removal of thrombus is possible. Also it can be used for immunoassay for specific detection of free TPA only. (12pp Dwg.No.0/0)

B(4-B2C3, 4-B4A3, 4-B4C5, 11-C7A, 12-K4A) D(5-H8, 5-H11) B0135

91-284374/39 B05 D16 E17 DAIL 15.12.89
DAICEL CHEM IND KK *JO 3187-399-A
15.12.89-JP-325360 (15.08.91) C12n-41

Optically active 1,3-butene diol mfr. - by allowing microbe e.g. aciculoconidium etc. to act on enantiomer mixt. of 1,3-butene diol and extracting (R)-1,3-butene diol

C91-123116

Mfg. optically active 1,3-butane diol comprises (1) allowing one of the microbes which belong to Aciculoconidium, Brettanomyces, Cochliobolus, Corynespora, Dactylium, Echinopodospora, Hamigera, Helminthosporium, Nectria or Phialocephala and which have the properties of acting on theenantiomer mixt. of 1,3-butane diol and of allowing (R)-1,3-butane diol to survive, to act on the enantiomers- mixt. of 1,3-butane diol and (2) extracting optically active (R)-1,3-butane diol. Pref. another method comprises (11) allowing one of the microbes which belong to Ambrosiozyma, Bordetella, Fusarium, Gibberella, Glomerella, Gonatobotryum, Neosartorya, Oospora, Paecilomyces, Preussia, Spetoria, Talaromyces or Westerdykella and which have the ' properties of acting on the enantiomer mixt. of 1,3-butane diol and allowing (S)-1,3-butane diol to survive, to act on the enantiomer mixt. of 1,3-butane diol and (12) extracting optically active (S)-1,3-butane diol.

USE/ADVANTAGE - Optically active 1,3-butane diol high in optical purity is mfd. (6pp Dwg.No.0/0)

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54 Title of Invention: Monoclonal antibodies for human Interleukin-4, and methods for using the antibodies.

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Specifications

1. Title of the invention.

Monoclonal antibodies for human Interleukin-4, and methods for using the antibodies.

2. Claims.

1) Mouse monoclonal antibodies, 380-1 and 144-6, which have high affinity and specifically recognize human Interleukin-4.

2) A method of measuring human Interleukin-4 in a sample, the method characterized by the following steps:

(1) A sample containing human Interleukin-4 is added to a monoclonal antibody 380-1 or 144-6 (first antibody) for solid-phase Interleukin-4, and the monoclonal antibody and Interleukin-4 are bound;

(2) The combination obtained in (1) is bound to a polyclonal antibody (second antibody) for human Interleukin-4;

(3) The second antibody is recognized in the combination obtained in (2), and an antibody which is labeled with a marker (third antibody) is added;

(4) The marker's activity is measured.

3. Detailed explanation of invention.

[Industrial field of application]

This invention relates to monoclonal antibodies which have high affinity and specifically recognize human Interleukin-4 (hereafter IL-4), and to methods of using those monoclonal antibodies, in particular an immunoassay using those antibodies.

[Background of the invention]

IL-4 is a glycoprotein, molecular weight 18,000~21,000, produced by the T-lymphocytes in response to stimulus from lectin or phorbol ester, or from antigens. It has various functions which are critical to the body's immunological reactions, including differentiation and growth of B-lymphocytes, differentiation and growth of T-lymphocytes, and differentiation and growth of mastocytes [See Y. Noma et al, Nature 319 640-646 (1986); F. Lee et al, Proc. Natl. Acad. Sci. USA 83 2061-2065 (1986); E. Severinson et al, Eur. J.

Immunol. 17 67-72 (1987); and T. R. Mosmann et al, Proc. Natl. Acad. Sci. USA 88 5654-5658 (1986)]. The cDNA of human IL-4 was cloned in 1986, and its amino acid sequence was described as follows [See T. Yokota et al, Proc. Natl. Acad. Sci. USA 83 5894-5898 (1986)].

MetGlyLeuThrSerGlnLeuLeuProProLeuPhePheLeuLeuAlaCysAlaGly
AsnPheValHisGlyHisLysCysAspIleThrLeuGlnGluIleIleLysThrLeuAsnSerL
euThrGluGlnLysThrLeuCysThrGluLeuThrValThrAspIlePheAlaAlaSerLys
AsnThrThrGluLysGluThrPheCysArgAlaAlaThrValLeuArgGlnPheTyrSerH
isHisGluLysAspThrArgCysLeuGlyAlaThrAlaGlnGlnPheHisArgHisLysGlnL
euIleArgPheLeuLysArgLeuAspArgAsnLeuTrpGlyLeuAlaGlyLeuAsnSerCy
sProValLysGluAlaAsnGlnSerThrLeuGluAsnPheLeuGluArgLeuLysThrIleM
etArgGluLysTyrSerLysCysSerSer.

It has recently been discovered that IL-4 functions to induce production of IgE in the B cells, and to stimulate the growth of mastocytes [see J. Exp Med. 169 (4), 1295 (1989)], and It is thought that IL-4 may be involved in allergic reactions.

Since detailed study of the physiological functions of human IL-4 has barely begun there are great expectations for future research achievements, but to achieve further advances it is urgently necessary to establish a microassay method for IL-4.

Conventionally, IL-4 has been measured by means of its bological functions. Namely, the amount of IL-4 produced is estimated based on the degree of T-cell activity caused by the IL-4. This method is called bioassay. However, ecause the T-cells are activated by internal factors other than IL-4, the detection precision of bioassay is reduced, he detection limit is low (about 250pg/ml), and there are severe limits on the use of this method.

[Conventional technology]

Within the industry, immunoassay is regarded as a method with greater precision than bioassay and a superior detection limit. In order to achieve immunoassay, it is necessary to obtain

monoclonal antibodies which have high affinity and specifically recognize IL-4.

Up to now there have been three reports, as described below, relating to monoclonal antibodies for IL-4 and/or to immunoassay using such antibodies.

(1) European Patent Disclosure #314402 reported on rat monoclonal antibodies for human IL-4, and an immunoassay by the sandwich technique using those monoclonal antibodies. In detail, the rats were first sensitized by an ordinary method using intraperitoneal administrations of recombinant human IL-4. Then their spleens were removed, and the collected spleen cells were fused with mouse myeloma cells. The resulting hybridoma group was screened by an ordinary method to obtain the cells IC1.11B4.6 and MP4.25D2.11, which produce antibodies which bind specifically to human IL-4. The class of the monoclonal antibodies produced by IC1.11B4.6 was an isotype of rat IgG_{2a}, and the class of the monoclonal antibodies produced by MP4.25D2.11 was IgG_1. An immunoassay for human IL-4 was also achieved using those monoclonal antibodies. Using that method, it was possible to detect up to 50pg/ml of IL-4 in human serum.

(2) A measurement reagent kit for human IL-4 which uses the sandwich technique is already being marketed by Genzyme Corp. The character of the monoclonal antibodies used in the kit and the method used to obtain them are not disclosed in the manual, but we have inferred from a confirmation test that mice were used as the sensitization animals. We could not confirm the class of antibody, but we did confirm that it completely blocks human IL-4 activity at a

concentration of a 6/1 dilution of liquid concentrate (concentration unknown) (see Figure 2 (b)). The manual also states that the detection limit of the kit is 90pg of human IL-4/ml.

Patent Disclosure #1991-187395 (3)

(3) Biochem, J., 262 897 (1989) describes the mouse monoclonal antibodies 4B2-F9 and 4B2-H12 for human IL-4. The class of 4B2-F9 is an IgG_1 isotype, and it blocks almost 100% of human IL-4 activity at a concentration of 6.25μg/ml. 4B2-H12 is an IgG_2 isotype, and in a human IL-4 receptor binding assay using ^{125}I-recombinant human IL-4, it had no effect up to a concentration of a 7/1 dilution of peritoneal fluid, according to the article.

[Problems with conventional technology]

The detection limit for already known human IL-4 immunoassays is on the order of 10^{-11}g/ml. Considering that human IL-4 is secreted in the body in minute amounts, this value is insufficient.

[Methods for solving the problems]

As a result of painstaking research aimed at achieving an immunoassay method with superior precision and detection limit, the inventors have obtained monoclonal antibodies which have high affinity and specifically recognize human IL-4. We found ourselves within sight of our goal and perfected this invention.

It is clear from their characteristics that the monoclonal antibodies of this invention are new and completely different from the three monoclonal antibodies described under [Conventional technology]. Namely, the monoclonal antibodies of this invention are mouse antibodies obtained by sensitizing a mouse with human

IL-4, and their class has been confirmed as IgG_1, K isotype. In contrast, the antibodies described in (1) under [Conventional Technology] were obtained from rats, so the two are fundamentally different. In (2), although the sensitized animal is not disclosed in the manual, we inferred from our confirmation test that it was a mouse. However, the two antibodies have different blocking patterns for human IL-4. Namely, the monoclonal antibody 380-1 of this invention blocks about 70% of human IL-4 even at a concentration of 100μg/ml, and never blocks 100% of activity. In contrast, the monoclonal antibody described in (2) was shown to block 100% at a concentration of a 6/1 dilution of liquid concentrate (concentration unknown). This means that the two antibodies have different recognition sites for human IL-4, and thus that they have different hypervariable regions. Consequently, we can conclude that the monoclonal antibody 380-1 of this invention has an entirely different structure from the monoclonal antibody described in (2).

The other monoclonal antibody included in this invention, 144-6, is an IgG_1, K isotype antibody. Since it blocks only 20% of human IL-4 activity eve at 100μg/ml, we can say that it is structurally different from the antibody described in (2) under [Conventional technology].

The antibodies described in (3) are also mouse antibodies, but they too are different from the monoclonal antibodies of this invention both in class and blocking pattern. Namely, while the antibody 4B2-F9 blocked IL-4 activity almost 100% at 6.25μg/ml, the antibody 380-1 of this invention blocks it only 70+% at a

concentration of 100μg/ml. And while 4B2-H12 is an IgG_2 isotype, the antibody 144-6 of this invention is an IgG_1, K isotype. Furthermore, in a human IL-4 receptor binding assay using ^{125}I-recombinant human IL-4, it was reported that 4B2-H12 had no effect up to a concentration of a 7/1 dilution of peritoneal fluid (estimated to be about 1000μg/ml), while the antibody 144-6 of this invention has 82% binding blocking activity at 100μg/ml.

Furthermore, the detection limit for assays of (1) and (2) under [Conventional technology] was 10^{-11}g/ml, while that for this invention was 3.9 pg/ml, or a measurement capability on the order of 10^{-12}g. We could not have predicted that using the monoclonal antibodies of this invention would elevate the detection limit by one order of magnitude.

[Description of the invention]

This invention consists of the mouse monoclonal antibodies

380-1 and 144-6, which have high affinity and specifically recognize human IL-4, and of methods of using these monoclonal antibodies.

Of the antibodies of this invention, 380-1 blocks 72.5% of the biological activity of human IL-4 at a concentration of 100μg/ml, and even when the concentration of antibodies is increased still further blocking does not rise to 100%. 144-6 blocks only 20% even at a concentration of 100μg/ml.

In an IL-4 receptor binding assay using ^{125}I-human recombinant IL-4, 380-1 has binding blocking activity of 94.5% at

100μg/ml, while 144-6 has binding blocking activity of 82% at 100μg/ml.

Consequently, we believe that these 2 kinds of antibodies recognize different epitopes of human IL-4. It has also been confirmed that the IgG subclass of both antibodies is mouse IgG_1, K.

The monoclonal antibodies of this invention can be prepared in the following way:

(1) Sensitize mice using human IL-4 as the immunogen;

(2) Fuse the spleen cells of the sensitized mice with mouse myeloma cells;

(3) Screen the cells which produce monoclonal antibodies for IL-4 from the resulting hybridoma;

(4) Clone the desired antibody-producing hybridoma;

(5) Reproduce the cloned antibody-producing hybridoma;

(6) Isolate and refine the produced antibodies.

The steps are explained in more concrete detail below.

The immune sensitization process (1) consists first of an initial immune sensitization stage in which human IL-4 (either natural or manufactured by gene manipulation) is dissolved in phosphate buffer containing physiological salt (hereafter PBS), emulsified with Freund's complete adjuvant (FCA) at a ratio of 1:1 and then administered to mice intraperitoneally. Two weeks later, PBS containing human IL-4 is similarly emulsified with Freund's incomplete adjuvant (FICA) at a ratio of 1:1 and administered intraperitoneally, and two weeks after that, PBS containing human IL-4 is administered intraperitoneally to complete the process. The type of mice used is not limited, but BALB/c is ideal. The number of

sensitizations and the dosage of antigen are not limited, but three administrations of 50~100μg of human IL-4 each time is sufficient.

In step (2), cell fusion is performed by first removing the spleens of the mice which were immune sensitized in step (1). A suspension of spleen cells is prepared according to ordinary methods, and a mixture of spleen cells and mouse myeloma cells is next obtained, to which is added polyethylene alcohol (preferably PEG 1500) at a temperature of 37°C. Many kinds of mouse myeloma cells are known, including P3 X 63Ag8, P3/NS1/1-Ag4-1, and SP-2/0-Ag-14, all of which can be easily obtained. An HGPRT (hypoxanthine guanine phosphoribosyl-transferase) defective cell strain which cannot live in HAT medium (medium containing hypoxanthine, aminopterin and thymidine) is useful for the myeloma cells, and also it is better that the myeloma cell strain be one that does not itself secrete antibodies. Ideally, SP-2/0-Ag-14 is used.

Next, the resulting mixture of fused cells is divided at low cell density into 96 microwell plates and cultured in HAT medium. During 1~2 weeks of cultivation the unfused myeloma cells, hybridoma of myeloma cells only, and unfused spleen cells will die off because their living conditions are not fulfilled, and only the hybridoma of spleen cells and myeloma cells will continue to grow.

In step (3), screening is carried out by measuring the anti-human IL-4 activity of hybridoma culture supernatant. Namely, rabbit polyclonal antibodies (IgG [intersection? subclass? ?]) for mouse IgG are first bound to dead yellow staphylococcus,

then hybridoma culture supernatant and human IL-4 are added. IL-4 activity in the supernatant is measured by bioassay, and a specimen in which activity has decreased or fallen to zero can be judged to be producing monoclonal antibodies.

Step (4) is carried out by cloning the antibody-producing hybridoma according to the soft agar culture method (see Monoclonal Antibodies, p. 372 (1980)). Limited dilution analysis can also be used at this stage.

Step (5) can be achieved by culturing the cloned hybridoma in ordinary medium and then separating and refining the culture supernatant. However, a method used to obtain antibodies more efficiently and in larger quantities is to administer the hybridoma to mice peritoneally, let it grow, and separate and refine it from the peritoneal fluid.

In step (6), the antibodies can be refined by ordinary methods, for example by salting out, ion exchange chromatography, gel filtration, hydrophobic chromatography, or affinity chromatography. However, a more effective method is affinity chromatography using Affigel Protein A Maps II column (Bio-Rad Corp.).

Because the monoclonal antibodies of this invention have high affinity and specifically recognize human IL-4, they can be used in refining human IL-4, for example in affinity chromatography.

Furthermore, of the antibodies of this invention, antibody 380-1, which blocks the activity of human IL-4, can be used either by itself or as a chimera antibody with human IgG to treat or prevent various diseases which are thought to involve abnormal IL-4

production, for example those diseases whose causes are attributed to I-type allergies, such as anaphylactic shock, rashes, asthma, rhinitis and intestinal allergies, as well as autoimmune diseases such as chronic rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, and myasthenia gravis.

However, the greatest and main use for the monoclonal antibodies of this invention is their application in human IL-4 immunoassays which are precise and have high detection limits.

Among immunoassays, both the one-point binding [?] measurement method and the two-point binding measurement method (or sandwich method) are well known, but the two-point binding measurement method is superior from the standpoint of precision and detection limit.

Two-point binding measurement is a method of measuring human IL-4 in a sample according to the following steps, as shown. Figure 1 shows an outline of the method.

(1) Add a sample containing human IL-4 to monoclonal antibodies (first antibodies) for solid-phase human IL-4, and bind the monoclonal antibodies and the human IL-4;

(2) Bind the combination obtained in step (1) with polyclonal antibodies (second antibodies) for human IL-4;

(3) Recognize the second antibody in the combination obtained in step (2), and bind an antibody which is labeled with a marker (third antibody);

(4) Measure the activity of the marker.

Either of the antibodies 380-1 or 144-6 of this invention can be used as the first antibody, but 380-1 is preferable. The solid phase and immobilization methods used in immunoassay are well known (see Immobilized Enzymes, Ichiro Senhata ed., Kodansha, 1975). For example, for the solid phase, polystyrene plate, polystyrene beads, nylon beads, glass beads, protein G agarose beads or polystyrene tubes are used, but the marketed 96 well polystyrene plate is preferable. An insoluble method using either physiological adhesion or covalent bonding can be used for immobilization. The reactions between the first antibodies and human IL-4 in the sample take place when they are left overnight at 24°C.

If the second antibody is a polyclonal antibody for human IL-4, there are no limits on the type of sensitized animal. The polyclonal antibody can be made using well-known methods. For example, human IL-4 (natural

or manufactured by gene manipulation) mixed with a suitable adjuvant is administered to animals (for example rats, mice, marmots, rabbits, cats, dogs, sheep or goats, preferably rabbits) several times at suitable intervals, either intravenously, subcutaneously or peritoneally, in order to sensitize them. After sensitization, serum is extracted, then separated and refined by affinity chromatography (or another method), and the polyclonal antibody for human IL-4 is manufactured by obtaining the desired antibody [subclass?]. The reaction between the first antibody IL-4

combination and the second antibody takes place when they are left at 24°C for several hours, preferably two hours.

There are no particular limits on the third antibody as long as it is one that recognizes the second antibody. Enzymes are commonly used as markers, but radio isotopes or fluorescent materials can also be used. The enzymes can be any enzymes which are normally used in enzyme immunoassay, for example peroxidase, ß-D-galactosidase, alkaline phosphatase, glucose-6-phosphodehydrogenase, or alcohol dehydrogenase; horseradish peroxidase is preferable. Goat polyclonal antibodies for rabbit IgG, which have been marked with peroxidase and similarly marked sheep polyclonal antibodies are marketed. Even those not marketed can easily be manufactured by well-known methods. The reaction between the third antibody and the first antibody--IL-4--second antibody combination takes place when they are left for several hours - preferably 2 hours - at 24°C.

Measurement of marker activity in step (4) is also carried out according to well-known methods. For example, if the third antibody has been marked with peroxidase, measurement can be carried out using an [orthophenylendiamin] substrate by causing a hydrogen peroxide reaction and then measuring the O.D. 490 of the reaction products. In this case, 3-(4-hydroxiphenyl) propionic acid or 3,3',5,5'-tetramethylbenzene can also be used as the substrate. In other cases, an appropriate substrate can be used.

A more convenient method of two-point binding is known in which the second antibody is labeled with a marker, eliminating the reaction with the third antibody. In this case, the polyclonal

antibody for IL-4 can itself be marked, or else the antibody can be split by papain to form Fab fragments, or split with pepsin to form $F(ab')_2$ fragments, or such fragments can be split reductionally to form Fab' fragments. The fragments are then marked and used for the second antibody.

Another method of applying the two-point binding method just described is to use a polyclonal antibody for human IL-4 as the first antibody and a monoclonal antibody for human IL-4 as the second antibody, and measure with the third, marked antibody (for example, a polyclonal antibody for mouse IgG). Using the same method, it is also possible to mark the second antibody itself, or a Fab fragment, $F(ab')_2$ fragment or Fab' fragment of it, thus eliminating the reaction with the second antibody.

The monoclonal antibodies of this invention can also be used in assays of human IL-4 using one-point binding.

One-point binding measurement consists of the following steps:

(1) A sample containing human IL-4 is immobilized into the solid phase;

(2) A monoclonal antibody for human IL-4 (first antibody) is added and bound with the human IL-4;

(3) In the combination obtained in (2), the first antibody is recognized, and a polyclonal antibody labeled with a marker is bound;

(4) The activity of the marker is measured.

The solid phase, immobilization method, marker and reaction conditions can be selected at will, as in two-point binding.

[Effects of the invention]

By using the monoclonal antibodies for human IL-4 of this invention, 380-1 and 144-6. we achieved an immunoassay for human IL-4 which is extremely sensitive (measurement limit 3.9 pg/ml), and has great specificity and reproducibility.

Patent Disclosure 1991-187395 (7)

[Working examples]

This invention is explained more concretely below using working examples. However, these examples do not limit the scope of this invention.

Working example 1

Manufacturing the monoclonal antibodies 380-1 and 144-6 for human IL-4.

(1) Mouse sensitization.

An emulsion consisting of FCA (0.5m.) and PBS (0.5ml) containing 50Mg of recombinant human IL-4 (made according to the method recorded in the specifications for patent application #1988-166871) was administered peritoneally to two BALB/c female mice. Two weeks later, an emulsion prepared as before and consisting of FICA (1:1) and recombinant human IL-4 dissolved in PBS was administered peritoneally as a booster. Two weeks after that, recombinant human IL-4 (80μg) dissolved in PBS (1ml) was administered peritoneally.

(2) Cell fusion

Three days after the last immunization, the spleens were removed from the sensitized mice and the spleen cells were prepared. The resulting spleen cells were mixed with mouse

myeloma cells [SP-2/0-Ag1M, prepared according to the method described in Nature, 276, 269 (1978)] at a ratio of 10:1. Polyethylene glycol [PEG 1500 (registered trademark), MA Bioproducts Co.] was added at a concentration of 50%, and cell fusion was carried out according to the Goding method [see J. Immunol. Methods, 39, 285 (1980)].

Following cell fusion, the cell mixture was suspended with Dulbecco's [modified?] Eagle's basal medium (hereafter DME) (4.5g/lit. glucose type, [Gibco?] Co.) containing 10% fetal bovine serum (FBS), 10% horse serum (HS), 10% NCTC109 medium (registered trademark, MA Bioproducts Co.), hypoxanthine (13.6μg/ml), thymidine (3.9μg/ml) and glycine (2.0μg/ml). The suspension was then divided into 96 wellplates and cultivated at 37°C in an atmosphere containing 7% CO^2. 2, 4 and 7 days after the start of culture, half of the medium was exchanged for HAT medium (the aforementioned Eagle's medium containing 0.18μg/ml of aminopterin), and the culture continued. Starting 10 days after the start of culture, colonies shaped liked grape clusters formed in several of the wells. Finally, hybridoma growth was confirmed in 1006 wells [well 1006?].

(3) Screening monoclonal antibody production strains.

Screening was carried out according to the method of Kaneko et al [described in J. Biol. Chem., 262, 6741 (1987)]. Namely, rabbit polyclonal antibodies (IgG [subclass?], 60μg) for mouse IgG were added to yellow staphylococcus (5μg) suspended in 20mH of [Trisoux?] hydrochloric acid buffer (pH8.0, 100μlit.), and the staphylococcus and antibodies were bound. Then the unbound

antibodies were eliminated by repeated washing with PBS and centrifugation (1500 X g. 10 minutes).

Hybridoma culture supernatant (100μlit.) was added to the resulting combination. After washing with PBS and centrigfugation, the resulting pellets were suspended recombinant human IL-4 (2ng/ml) dissolved in 100μlit. of RPMI-1640 (Nissui Co.) containing 10% FBS, and then incubated at room temperature. One hour later they were centrifuged and IL-4 activity was measured in the resulting supernatant. If activity had decreased or disappeared, it was judged that antibodies for human IL-4 were being produced in the well.

Human IL-4 activity was measured using human lymphocyte growth stimulus activity as the indicator. Namely, lymphocytes prepared from human peripheral blood were suspended in culture solution (RPMI-1640 containing 10% bovine fetal serum, 5 X 10^{-5}M 2-mercaptoethanol) containing phytohemagglutinin (PHA, 10μg/ml), and then cultured for 4~6 days at 37°C in the presence of 5% CO_2. After being sufficiently washed (three times or more) with culture solution, the lymphocytes were resuspended so that 5 X 10^4 were contained in 200μlit. of culture suspension, the test sample was added, and culture was continued for three more days. In the final 12 hours, after pulsing with tritium thymidine, uptake into the macromolecule [subclass?] was measured using liquid scintillation counter.

Patent Disclosure #1991-187395 (8)

(4) Culturing antibody-producing hybridoma cells.

Cells which were judged to be producing antibodies for human IL-4 (2 wells) were cloned by soft agar culture using Kennett's method (see Monoclonal Antibodies, p. 372 (1980)).

$1\ 0^7$ cloned cell strains were transplanted inside the abdominal cavities of BALB/c female mice which had first been [Pristan?] processed. About two weeks later, at the point when a large quantity of peritoneal fluid had accumulated, peritoneal fluid was extracted. After being demarcated with 50% saturated ammonium sulfate, the extract was refined by affinity chromatography using affigel protein A MapsII column (Bio-Rad Co.), to obtain the IgG [subclass?]. The hybridoma strains 380-1 and 144-6 which produce the monoclonal antibodies 380-1 and 144-6 of this invention were both deposited on June 21. 1989 at the Biseibutsu Kogyogijutsu Kenkyujo [Microbial Industrial Technology Laboratory?] under the [deposit numbers?] 2486 (FERM BP-2486) and 2485 (FERM BP-2485).

Working example 2

Characteristics of the monoclonal antibodies of this invention.

(1) Immunoglobulin subclass

For the monoclonal antibodies 380-1 and 144-6 manufactured in working example (1), subclass was screened using a mouse Mono Ab-ID EIA kit (Zymed Co.). The results showed that both antibodies were mouse IgG_1, κ.

(2) Biological characteristics

We investigated the effects of both antibodies on the activation of IL-4 for cells (PBA) which had been subject to blastogenesis by the PHA of human peripheral lymphocytes. Namely, we mixed recombinant human IL-4 (2.5ng/ml) and

monoclonal antibodies at various concentrations, and cultured them along with PBL 5 X 10^4/0.2ml/well at 37° in atmosphere containing 5% CO_2 for 72 hours. After culture the biological activity of the PBL was measured using the MTT method (see Medical Immunology, 12, 411 (1988)). The results are shown in Figure 2 (a). The figure shows that monoclonal antibody 380-1 blocked human IL-4 activity beginning at 0.1μg/ml, and blocked 72.5% at 100μg/ml, but never blocked activity completely. On the other hand, monoclonal antibody 144-6 blocked only about 20% even at 100μg/ml. This probably means that 380-1 and 144-6 recognize different parts of the solid structure of human IL-4. Furthermore, 380-1 may not entirely recognize the part of human IL-4 which binds with receptors and transmits signals. Rather, the blocking effect results when it either causes changes in the solid structure of human IL-4 by binding with human IL-4, or when it blocks binding to the receptors.

(3) Western blotting

From the contrast between SDS-Page and the culture supernatant of recombinant human IL-4-producing CHO cells (see Patent Application 1988-186871), in western blotting carried out using 380-1 and 144-6 (according to the method in Proc. Natl. Acad. Sci. U.S.A., 76, 4350 (1979)), several bands were discovered in the molecular weight 14~19Kd region. Their locations corresponded to those of bands discovered by western blotting carried out in the same way using refined human IL-4. Furthermore, using this method, the presence of 0.2ng of recombinant human IL-4 was confirmed with 380-1, and 5ng with 144-6.

(4) Human IL-4 receptor binding assay

Human recombinant IL-4 was iodinized (^{125}I) with Iodogen reagent (Pierce Co.) using the Fraker method [described in Biochem. Biophys. Res. Comm., 80. 849 (1978)], to obtain a specific activity 4 X 10^{15} cpm/mmol labeled compound.

150pM of ^{125}I - human recombinant IL-4 were added in the presence of 10^6 PHA stimulus human peripheral lymphocytes and each concentration of antibodies or 3μM of unmarked IL-4, and then

with a final volume of 200μlit. they were incubated for two hours at 4°C using RPMI-1640 medium (pH 7.2) containing 10% FBS, 20aH HEPES and 0.2% sodium alginate.

The bound and unbound ^{125}I-IL-4 were separated by the oil cushion method [described in Nature, 320 75 (1988)] and radiation activity was measured using a γ-counter (Shimazu Manufacturing). The radiation activity values in the presence of [excessive?] IL-4 were subtracted from each measurement as the non-specific binding value, to calculate the specific binding value. Figure 3 shows the results. The figure shows that the monoclonal antibodies 380-1 and 144-6 of this invention dose-dependently block binding with IL-4 receptors beginning at 0.3μg/ml. At 100μg/ml, 380-1 showed 94.5% blocking activity and 144-6 showed 82%. In this experiment, antibodies unrelated to IL-4 (monoclonal antibodies for cow insulin) were used as a control, and had no effect even at 100μg/ml.

Working example 3

Immunoassay using the monoclonal antibody 380-1 of this invention.

(1) Preparation of rabbit polyclonal antibodies for human IL-4.

An emulsion consisting of FCA (1 ml) and PBS (1 ml) containing 2 mg of recombinant human IL-4 (manufactured by the method described in the specifications of Patent Application 1988-186871) was administered subcutaneously in several places on the backs of male New Zealand white rabbits. 10, 24 and 38 days after the initial sensitization, an emulsion prepared as before consisting of FICA (1:1) and PBS containing recombinant IL-4 was administered subcutaneously as a booster. 58 days after the first sensitization, final sensitization was carried out using recombinant IL-4 (3mg) dissolved in 3 ml of PBS. Seven days later all the blood was extracted through the carotid, left at room temperature for three hours, and centrifuged (1500 X g, 10 minutes) to obtain serum. The resulting serum was refined by affinity chromatography using Protein A Sepharose CL-4B column (Pharmasia Co.) to obtain IgG [intersection? subclass? ?]. The [subclass?] was dialyzed for PBS to obtain rabbit polyclonal antibodies for human IL-4.

(2) Preparing the human IL-4 immunoassay reagent.

(a) First antibody solution.

The monoclonal antibody of this invention (380-1, prepared in working example 1) was prepared in 0.1 M of carbonate-bicarbonate solution to a concentration of 30μg/ml.

(b) Second antibody solution.

Rabbit polyclonal antibodies for human IL-4 (prepared in working example 3-(1)) were prepared in PBS containing 2% FBS to a concentration of 2.5μg/ml.

(c) Third antibody solution.

Goat polyclonal antibodies (ZYMED Co.) for rabbit IgG marked with horseradish peroxidase were diluted 2,000 times with PBS containing 1% goat normal serum (Vector Co.).

(d) Standard solution.

Recombinant human IL-4 was prepared in RPMI-1640 containing 10% FBS to concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 pg/ml.

(e) Wash.

PBS containing 0.05% Tween 20.

(f) Blocking fluid.

Blocking reagent (Balinger Yamanouchi Co.) dissolved in 100ml of water. (Diluted 1:10 with water when used).

(g) Substrate solution.

(i) 0.03% hydrogen peroxide solution.

(ii) 10mg of [orthophenylendiamin?] hydrochloride tablets (hereafter OPD, Sigma Co.) were prepared in a 0.1 M citric acid / phosphoric acid buffer (pH 5.0) to a concentration of 2mg/ml.

(i) and (ii) are mixed in equal quantities before being used.

(h) Reaction arrest fluid.

1N sulfuric acid solution.

(3) Human IL-4 was measured using the reagent prepared in (2).

(1) The first antibody solution was added to 96 well immunoplates (Nunc Co.). 100μlit. per well, sealed, and left

overnight at 24°C. (The plates were also sealed in the instances below).

(2) The first antibody solution was collected from the plates and washed three times with wash (300μlit./well). (Washing was also carried out three times with 300μlit./well in the instances below).

(3) 300μlit. of blocking fluid were added to each well, and left for 4~5 hours.

(4) The blocking fluid was removed and washing carried out.

(5) 100μlit. of standard solution was added to each well and left overnight at room temperature.

(6) The standard solution was removed and washing carried out.

(7) 100μlit. of the second antibody solution was added to each well and left for 2 hours at 24°C.

(8) The second antibody solution was removed and washing carried out.

(9) 100μlit. of the third antibody solution was added to each well, and left for 2 hours at 24°C.

(10) The third antibody solution was removed, and washing carried out.

(11) 100μlit. of substrate solution was added to each well, and left to react for 10 minutes at 24°C (the reaction was carried out in a dark place).

(12) 100μlit. of reaction arrest fluid was added and the reaction stopped.

(13) After mixing for 30 seconds using a microplate mixer (Belco Co.), O.D.490 was measured in an [airizer?] (Japan Intermed Co.; Immunoleader-NJ-2000).

Table 1 shows the O.D.490 values of standard IL-4, and Figure 4 shows the values plotted as a standard calibration curve.

a Table 1: Standard IL-4 O.D.490 values in the assay method of this invention.

a [Top line]: Peroxidase activity

b [Lower line]: P value is for O.D.490 of IL-4 0ng/ml

Table 1 and Figure 4 show that using the assay of this invention concentrations of up to 3.9 ~ 500pg/ml of human IL-4 can be measured. The detection limit value of 3.9 pg/ml is much greater than the 50 pg/ml shown in (1) and the 90 pg/ml in (2) under [Conventional technology], indicating that this assay can stand up adequately to the demands of the field of ultramicroassay.

Working example 4

Characteristics of the immunoassay of this invention.

(1) Correlations between this assay and bioassay.

Human IL-4 volumes were measured in various kinds of specimens using the assay of this invention (measured using the method in working example 3) and bioassay (assay using the human lymphocyte growth stimulus activity described in working example 1-(3) as the indicator). Figure 5 shows the correlation between the two assay methods.

The regression line for the correlations is n=5, 4=0.998., y=0.987, x+0.968, indicating a good correlation between the two assay methods.

(2) Addition recovery test using the assay of this invention.

Various concentrations of recombinant IL-4 were added to solvent (RPMI-1640 medium containing 10%FBS) containing recombinant IL-4 (about 14 pg/ml), and the amount of IL-4 in each sample was measured according to working example 3. The following table shows the recovered amounts and recovery rates.

Table 2: Addition recovery test

a Added amount	b Measured amount	c Recovered amount	d Recovery rate

The table shows that for every concentration the recovery rate was greater than 84%. This shows that the assay method of this invention is extremely precise.

(3) Intra-assay variance test for the assay of this invention.

After creating a calibration curve following the method described in working example 3, IL-4 concentrations in each sample in the same plates (containing about 25, 50 or 100 pg/ml of recombinant human IL-4) were measured at n=5, and the mean, standard deviation and coefficients of variation (C.V.) were calculated. The following table shows the results.

Table 3: Intra-assay variance test

a Sample No.

The table shows that at all concentrations the C.V. was within 6%, which means that the assay method of this invention is extremely precise.

(4) Inter-assay variance test for the assay of this invention.

According to the method described in working example 3, with the calibration curve being created and corrected each time, the

concentrations of IL-4 in each sample (containing about 25, 50 or 100 pg/ml of recombinant human IL-4) were measured 5 times, and the mean, standard deviation and coefficient of variation (C.V.) were calculated. The following table shows the results.

D Table 4: Inter-assay variance test

a Sample No.•

The table shows that for all concentration the C.V. was within 5.4%, which means that the assay method of this invention is extremely precise.

(5) Serum concentration effect in the assay of this invention

Various concentrations of recombinant human IL-4 (3.9 ~250 ph/ml) were added in the presence of 5, 20, 40 or 100% human serum [RPMI-1640 medium (Nissui Co.) used as the solvent], and measured according to the immunoassay of this invention (the method described in working example 3). Table 5 and Figure 6 show the results.

E-1 Table 5: Human serum effect in the assay of this invention (n=3)

•a O.D. 490 (100% human serum)• b O.D. 490 (40% human serum)

E-2 Table 5 (Continued)

•a O.D. 490 (20% human serum)• b O.D. 490 (5% human serum)

In immunoassay, the concentrations of human serum in the samples often have an adverse effect on the sensitivity, precision, and detection limit of measurement. However, the assay of this invention was found to be completely unaffected by serum concentrations. Namely, as shown in Tables 5 & 6, although the strength of coloration decreases depending on serum

concentrations, a clear linear relationship was shown in all cases. Even when the amount of human IL-4 added was 3.9 pg/ml, a significant difference of $p<0.01$ was preserved for 0 pg/ml.

(6) Measurement of natural human IL-4 using the assay of this invention.

Blood was taken from the peripheral veins of two healthy people (A and B) in the presence of heparin, and lymphocytes were prepared using Lymphoprep (Daiichi Kagaku Manufacturing). 10^6 lymphocytes were cultured for 24 hours at 37°C in atmosphere containing 5% CO_2, in the presence of various mitogens [Concanavalin A (Con A, Sigma Co.), phytohemagglutinin-P (PHA-P, Difco Co.), A 23187 (Calbiochem Co.) and phorbol 12-[myristate?] 13-acetate (PMA, Funakoshi Co.). ASF 102 medium (Ajinomoto Co.) containing 1% normal human serum, 0.5% cow serum albumin, 50 mg/lit. transferin, 4.5 g/lit. glucose, 0.3 mg/lit. oleic acid and 0.3 mg/lit. palmitic acid was used as the medium. After culture, the IL-4 content of the serum was measured using the immunoassay of this invention. Table 6 shows the results.

Table 6: IL-4 content of human peripheral blood lymphocyte culture serum

Mitogen	IL-4 (pg/ml) produced	
	Donor A	Donor B
Not added		

IL-4 production was confirmed in culture supernatant stimulated by Con A, PHA-P and A 23187. Under PMA stimulus it was below the detection limit. Consequently, it was proved that, although it

consists of monoclonal antibodies manufactured using recombinant IL-4 as the antigen. the assay of this invention is capable of measuring natural human IL-4.

4. Brief explanation of drawings

Figure 1 shows an outline of the human IL-4 assay of this invention.

Figure 2 (a) and (b) are graphs showing the blocking effects on human IL-4 activity by the antibodies of this invention and conventional technology, respectively.

Figure 3 is a graph showing the blocking effect of the monoclonal antibodies of this invention on the binding between human IL-4 and receptors.

Figure 4 is a calibration curve for the human IL-4 assay of this invention.

Figure 5 is a graph showing the correlation between the human IL-4 of this invention and conventional bioassay.

Figure 6 is a graph showing the effect of serum concentrations in the human IL-4 assay of this invention.

Applicant: Ono Yakuhin Kogyo Inc.

Agent: Patent Attorney, Kunihisa Oya

G Figure 1 - Solid phase

a Marker

b Measurement

c {Antibody which recognizes second antibody (third antibody)

d {Polyclonal antibody for human IL-4 (second antibody)

e Human IL-4

f {Monoclonal antibody for human IL-4 of this invention (first antibody)

H Figure 2 (a)

a [Y axis] a Blocking activity (% of control)

[X axis]

b Concentration of the antibody

c ○: Antibody of this invention. 380-1

d □ Antibody of this invention. 144-6

I Figure 2 (b)

[Y axis] a Blocking activity (% of control)

[X axis] b Concentration of antibody

c Dilution of concentrate

d •: Monoclonal antibodies in Conventional Technology (2)

J Figure 3

○: a Antibody of this invention 380-1

•: b Antibody of this invention 144-6

△: c Antibody used as a control

[Y axis] d Blocking activity (%)

[X axis] e Concentration of antibody (μg/ml)

L Figure 5

[Y axis] a Values measured according to the assay of this invention

[X axis] b Values measured according to bioassay

Patent Disclosure #1991-187395 (15)

M Figure 6

[Y axis] a Peroxidase activity

b Human serum

Continuation of first page

5 1 Int. Cl.[5] Identification symbols Intra-agency file No.s

K Figure 4

a (Y-axis) : Peroxidase activity

(inset: Y-axis): b Peroxidase activity

A

Table 1

第1表：本発明の定量法における標準ＩＬ－４の O.D.490値

ＩＬ－４ (pg／ml)	a ペルオキシダーゼ活性 (O.D.490) (n- 3)			
	MEAN	S. D.	C. V. (%)	P
1000	2.185	0.022	1.0	<0.001
500	1.534	0.031	2.0	<0.001
250	0.924	0.013	1.4	<0.001
125	0.517	0.005	0.9	<0.001
62.5	0.299	0.007	2.2	<0.001
31.3	0.183	0.013	7.0	<0.001
15.6	0.124	0.007	5.6	<0.001
7.8	0.108	0.009	8.5	<0.001
3.9	0.095	0.004	3.7	<0.001
0	0.077	0.001	0.7	

b （P値はＩＬ－４ ０ｎｇ／mlのO.D.490 に対して）

B

Table 2

第２表：添加回収実験

ＩＬ－４ (pg／ml)			d 回収率 (%)
a 添加量	b 実測値	c 回収量	
0	14.2	−	−
3.9	17.5	3.3	84.6
7.8	21.5	7.3	93.6
15.6	28.3	14.1	90.4
31.2	44.2	30.0	96.2
62.5	75.4	61.2	97.9
125	132	117.8	94.2

C Table 3

第３表：アッセイ内変動試験

a 試料No	n	Mean±S.D. (pg／ml)	C.V.(%)
1	5	23.3±1.4	6.0
2	5	46.6±2.0	4.2
3	5	95.2±1.4	1.5

D Table 4

第４表：アッセイ間変動試験

a 試料No	n	Mean±S.D. (pg／ml)	C.V.(%)
1	5	24.1±1.3	5.4
2	5	49.3±1.3	2.6
3	5	98.7±1.0	1.0

E-1

Table 5

第５表：本発明の定量法におけるヒト血清の影響（ｎ－３）

IL-4 (pg／ml)	a O.D.490(100%ヒト血清の場合) MEAN	S.D.	C.V.(%)	b O.D.490(40% ヒト血清の場合) MEAN	S.D.	C.V.(%)
250	0.506	0.026	### 5.2	0.572	0.018	### 3.1
125	0.287	0.021	### 7.3	0.313	0.006	### 1.8
62.5	0.177	0.016	### 9.1	0.188	0.004	### 2.1
31.3	0.115	0.005	### 4.3	0.114	0.001	### 0.5
15.6	0.081	0.003	### 3.3	0.074	0.000	### 0
7.8	0.063	0.002	### 2.7	0.057	0.002	### 3.7
3.9	0.056	0.002	## 2.7	0.049	0.001	## 2.0
0	0.048	0.002	3.6	0.039	0.002	5.1

##：$p < 0.01$

###：$p < 0.001$

E-2

Table 5 [cont'd]

第５表（つづき）

IL-4 (pg／ml)	a O.D.490(20% ヒト血清の場合) MEAN	S.D.	C.V.(%)	b O.D.490(5%ヒト血清の場合) MEAN	S.D.	C.V.(%)
250	0.686	0.033	### 4.8	0.871	0.036	### 4.1
125	0.395	0.022	### 5.5	0.501	0.023	### 4.5
62.5	0.227	0.005	### 2.0	0.280	0.011	### 3.8
31.3	0.134	0.004	### 3.3	0.164	0.004	### 2.2
15.6	0.093	0.001	### 1.2	0.106	0.003	### 2.4
7.8	0.069	0.002	### 2.5	0.075	0.001	### 1.3
3.9	0.059	0.002	### 2.9	0.061	0.002	### 3.3
0	0.047	0.002	3.2	0.041	0.002	3.7

##：$p < 0.01$

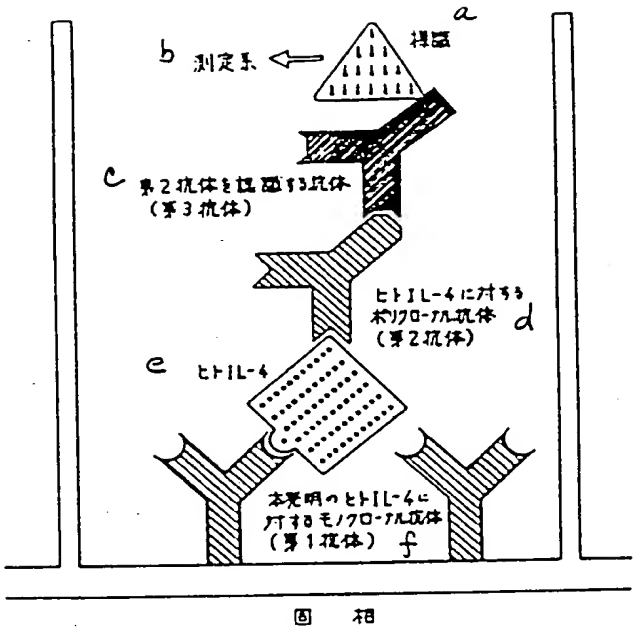
###：$p < 0.001$

F

Table 6

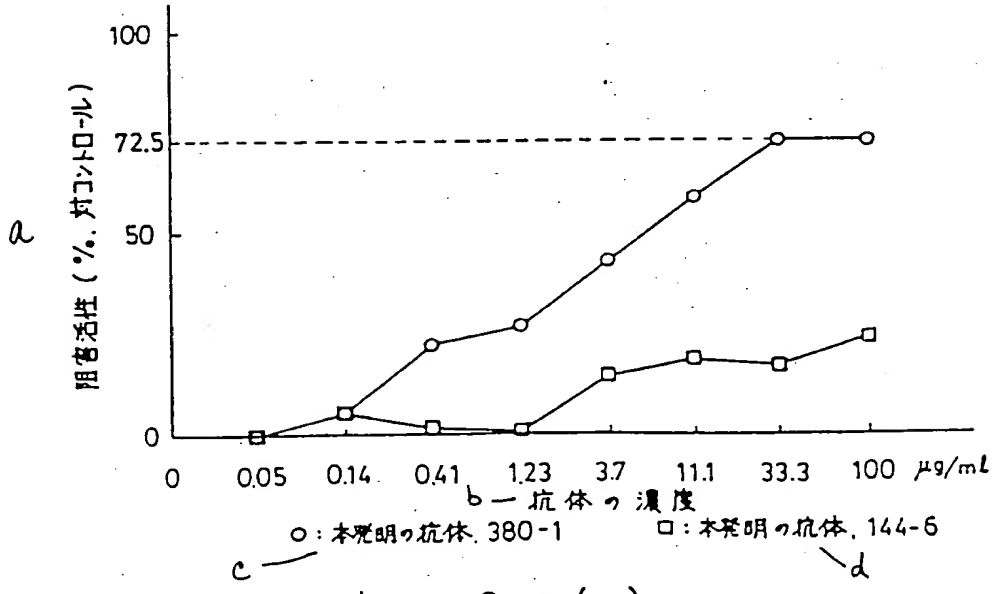
第６表：ヒト末梢血リンパ球培養上清中のＩＬ－４含量

d マイトジェン	a 生成したIL-4(pg/ml) b 供血者A	c 供血者B
e 無添加	<3.9	<3.9
Con A(10μg／ml)	57.5	52.3
PHA-P(10μg／ml)	53.5	59.8
A23187(200ng／ml)	52.5	31.5
PMA （２ng／ml）	<3.9	<3.9



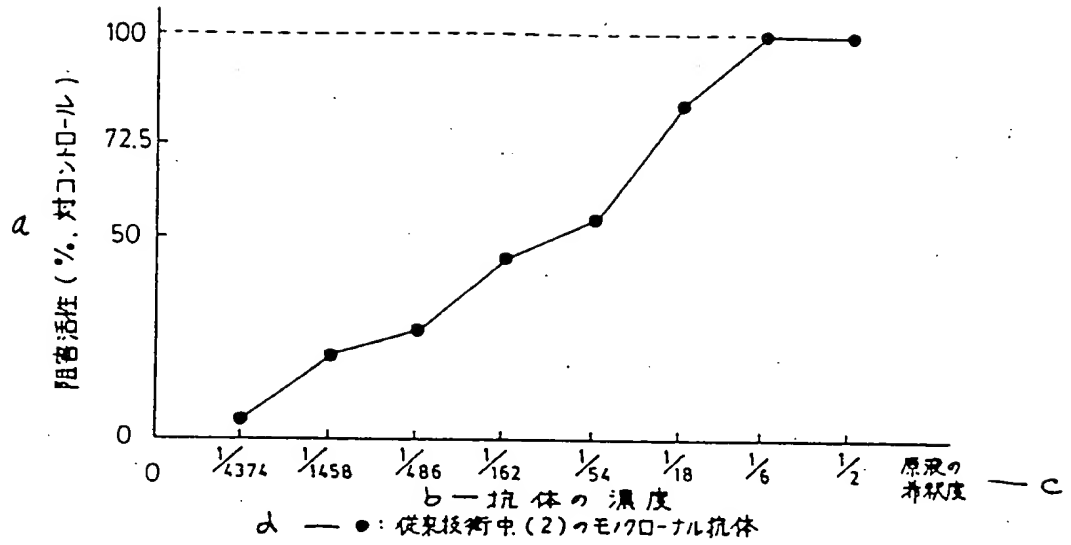
G 第1図

Figure 1



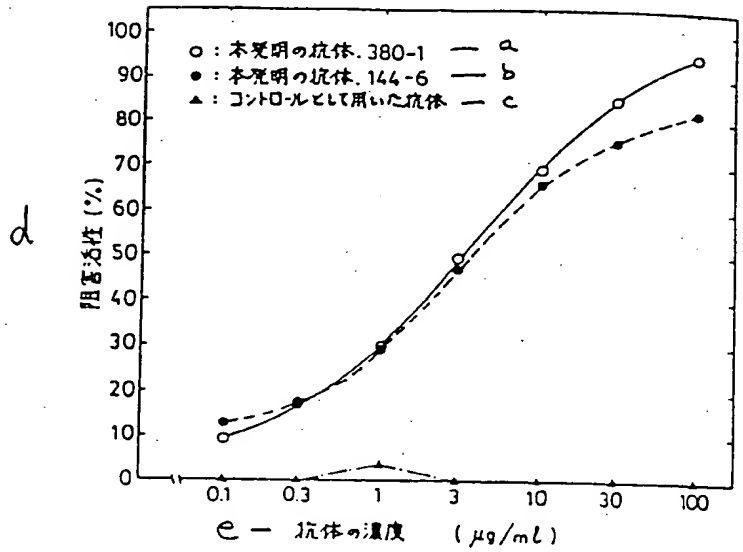
H 第2図(a)

Figure 2a



工　第 2 図（b）

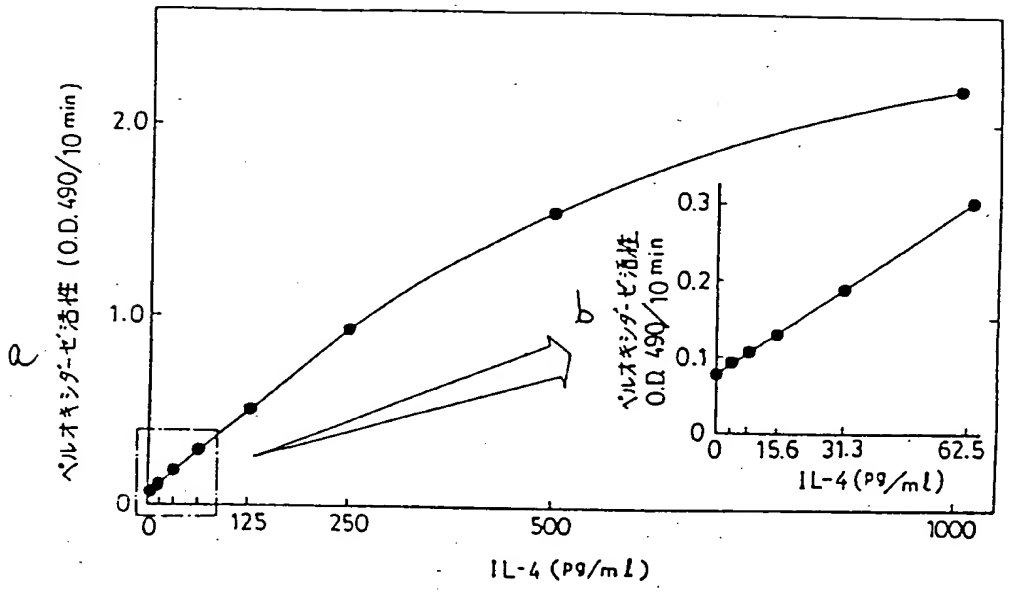
Figure 2 b



d

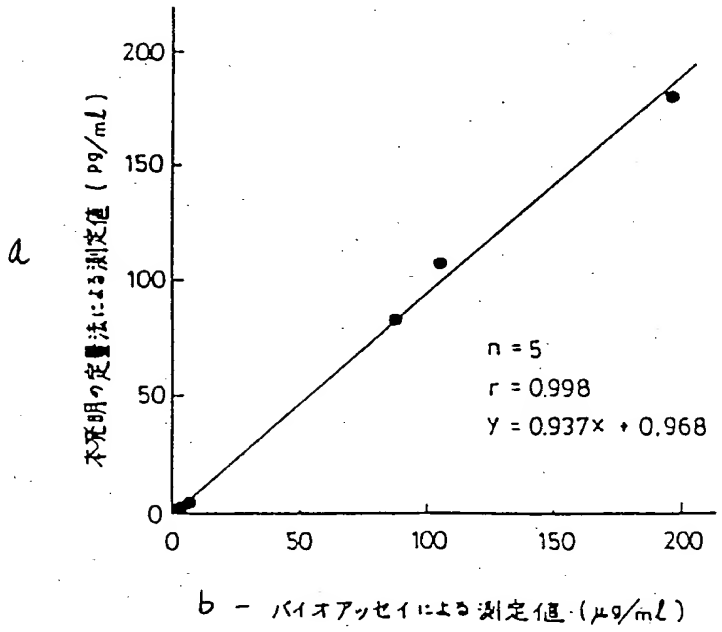
丁　第 3 図

Figure 3



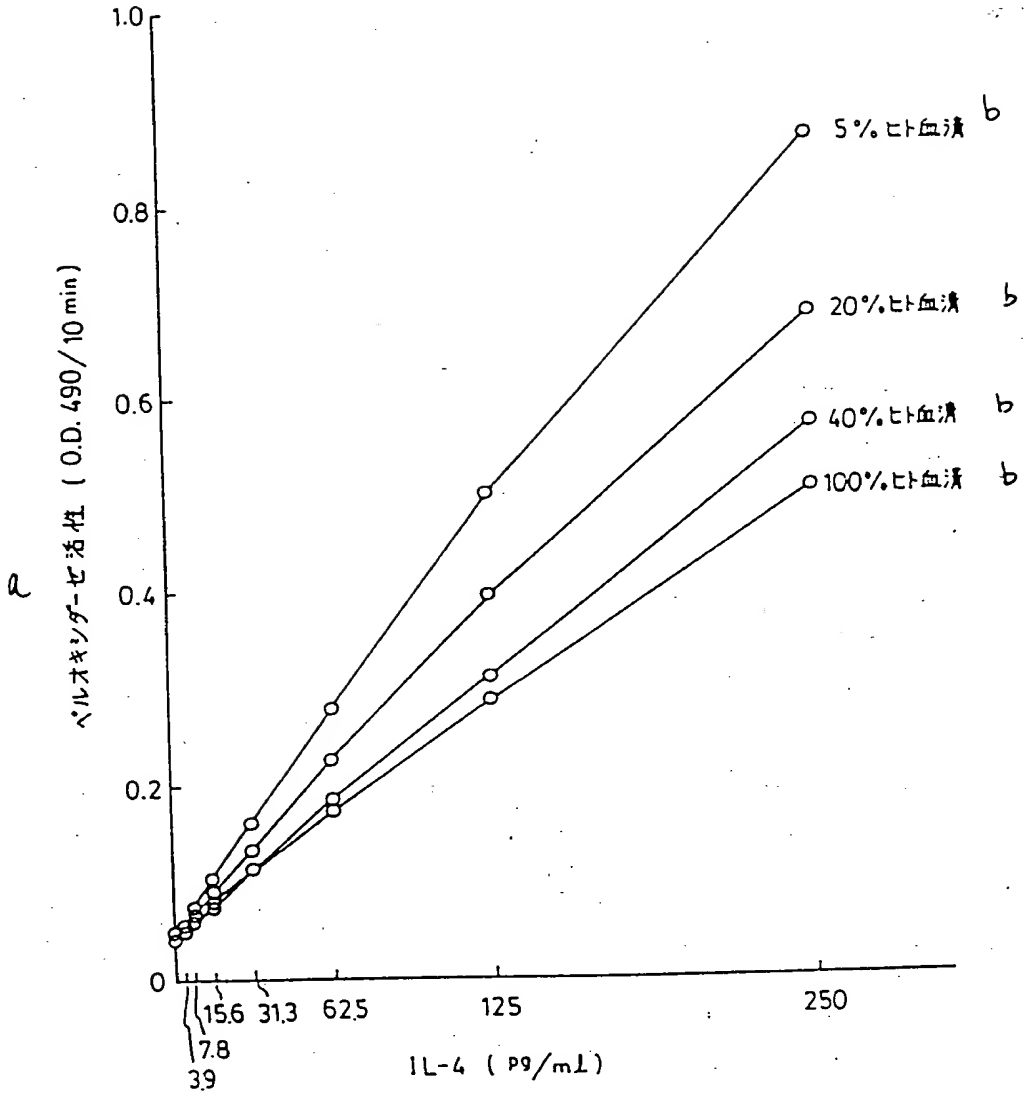
K 第 4 図

Figure 4



L 第 5 図

Figure 5


1.0
0.8
0.6
0.4
0.2
0
ペルオキシダーゼ活性 (O.D. 490/10 min)
5% ヒト血清
20% ヒト血清
40% ヒト血清
100% ヒト血清
15.6
31.3
62.5
125
250
7.8
3.9
IL-4 (pg/mL)

第 6 図

Figure 6